

# Identification of New Autoantigens by Protein Array Indicates a Role for IL4 Neutralization in Autoimmune Hepatitis\*<sup>§</sup>

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Autoimmune hepatitis (AIH) is an unresolving inflammation of the liver of unknown cause. Diagnosis requires the exclusion of other conditions and the presence of characteristic features such as specific autoantibodies. Presently, these autoantibodies have relatively low sensitivity and specificity and are identified via immunostaining of cells or tissues; therefore, there is a diagnostic need for better and easy-to-assess markers. To identify new AIH-specific autoantigens, we developed a protein microarray comprising 1626 human recombinant proteins, selected *in silico* for being secreted or membrane associated. We screened sera from AIH patients on this microarray and compared the reactivity with that of sera from healthy donors and patients with chronic viral hepatitis C. We identified six human proteins that are specifically recognized by AIH sera. Serum reactivity to a combination of four of these autoantigens allows identification of AIH patients with high sensitivity (82%) and specificity (92%). Of the six autoantigens, the interleukin-4 (IL4) receptor fibronectin type III domain of the IL4 receptor (CD124), which is expressed on the surface of both lymphocytes and hepatocytes, showed the highest individual sensitivity and specificity for AIH. Remarkably, patients' sera inhibited STAT6 phosphorylation induced by IL4 binding to CD124, demonstrating that these autoantibodies are

functional and suggesting that IL4 neutralization has a pathogenetic role in AIH. *Molecular & Cellular Proteomics* 11: 10.1074/mcp.M112.018713, 1885–1897, 2012.

Autoantibodies specific for proteins or nonprotein antigens (dsDNA, snRNP, carbohydrates) are often the serological hallmark of autoimmune diseases. Autoantibodies can be simply an epiphenomenon secondary to a chronic inflammatory milieu (1), but they can also play a direct pathogenetic role, as antithyroglobulin antibodies do in Hashimoto's thyroiditis (2).

Autoimmune hepatitis (AIH)<sup>1</sup> is a chronic necro-inflammatory disease of unknown etiology that affects predominantly women with an incidence of 1 to 2 per 100,000 per year and a prevalence of 10 to 20 out of 100,000 (3, 4). AIH is subdivided into two major types on the basis of autoantibody reactivity (5). Antibodies to nuclei and/or to smooth muscle characterize type 1 AIH, whereas antibodies to a liver-kidney microsomal constituent define patients with type 2 AIH. Because the detection of these autoantibodies is done by means of immunofluorescence on rodent multi-organ sections (liver, kidney, stomach), there are problems with the standardization and interpretation of the immunostaining patterns (6). To overcome these methodological problems, the International Autoimmune Hepatitis Group established an international committee to define guidelines and develop procedures and reference standards for more reliable testing (7, 8). Although ELISA and bead assays with purified or recombinant autoantigens are under development (9), they actually represent a

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<sup>1</sup> The abbreviations used are: AIH, autoimmune hepatitis; AUC, area under the curve; DELFIA, Dissociation-enhanced Lanthanide Fluorescence ImmunoAssay; FNIII, fibronectin type III domain; HBV, hepatitis B virus; HCV, hepatitis C virus; HD, healthy donor; IL4R, interleukin-4 receptor; IMAC, immobilized metal ion affinity chromatography; MFI, mean fluorescence intensity; PAM, predictive analysis of microarray; ROC, Receiver Operating Characteristic.

complementary, rather than alternative, approach to traditional immunofluorescence. Moreover, serological overlap is frequently observed between AIH and other non-autoimmune liver diseases such as chronic viral hepatitis (10). Therefore, new, highly specific markers represent an unmet medical need for the more accurate diagnosis and classification of AIH.

Besides the potential diagnostic application, the discovery of novel AIH autoantigens could provide insights into the disease pathogenicity mechanism. Although some AIH target-autoantigens have been identified and characterized, little is known about their pathogenetic role, and other autoantigens are probably still unknown. Autoantibodies, to be considered pathogenetic, must have at least two features: (i) the target-autoantigen should be either expressed on the plasma membrane of target cells or secreted by cells (*i.e.* should be exposed to autoantibodies), and (ii) binding of the autoantibodies to the target antigen should disturb a cellular function directly or indirectly. A possible pathogenetic role in AIH has been put forward for autoantibodies specific for cytochrome P450 2D6 (CYP2D6) or Asialoglycoprotein receptor 1 (AGPR-1), which are both present on the hepatocyte cell membrane (10).

Protein microarrays are a powerful technology, as they allow the simultaneous screening of thousands of analytes (11). In the present study, to identify new autoantigens with potential diagnostic and/or pathogenetic roles in AIH, we printed a microarray with 1626 human proteins whose main feature was being either secreted or membrane associated (*i.e.* potentially exposed to autoantibody recognition). We used this microarray to screen panels of sera from patients with AIH and identified six new protein antigens that are recognized with high sensitivity and specificity. One of these six autoantigens is the interleukin-4 (IL4) receptor fibronectin type III (FNIII) domain of the IL4 receptor (CD124), and, interestingly, patients' autoantibodies specific for CD124 neutralize IL4 signaling, suggesting a possible pathogenetic role for IL4 neutralization in AIH.

## EXPERIMENTAL PROCEDURES

**Serum Samples**—Samples used for this study were collected in five different hospitals according to standard operating procedures: (i) IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; (ii) Hepatology Unit, University Hospital, Pisa, Italy; (iii) Sant'Orsola-Malpighi University Hospital, Bologna, Italy; (iv) Center for Autoimmune Liver Diseases, IRCCS Istituto Clinico Humanitas, Rozzano, Italy; and (v) Center for Systemic Manifestations of Hepatitis Viruses, University of Firenze, Italy. For the discovery phase, 203 sera were used (15 AIH, 78 healthy donor (HD), 110 hepatitis C virus (HCV)), whereas for the validation phase 174 sera were used (50 AIH, 50 HD, 50 HCV, 24 hepatitis B virus (HBV)). The Institutional Review Boards of these hospitals authorized the use of sera for research purposes.

**Human Proteins: Selection, Expression, and Purification**—Genes whose translated products carry a secretion signal peptide or at least one transmembrane domain were selected, cloned, and expressed in a high throughput system as histidine-tagged products as described elsewhere (12). A total of 1626 polypeptides were cloned and expressed in *E. coli*. Of these, 1121 were cloned as protein fragments and 505 as full-length proteins (Supplemental Tables S1 and S2). The

recombinant proteins were affinity-purified from the bacterial insoluble fraction by means of immobilized metal ion affinity chromatography (IMAC) (GE). Representative SDS-PAGE gels of a panel of purified proteins are shown in Supplemental Fig. S1.

Human, viral, or bacterial proteins were used as biological or technical controls in the microarrays (Supplemental Fig. S2). HCV core protein and nonstructural proteins NS3 (from HCV genotype 1), NS3–4a (from HCV genotype 2), and NS5b (from HCV genotype 1); Tetanus toxin; and H1N1 antigen were produced in house by subcloning the corresponding genes in *E. coli* strain DH5 $\alpha$  and expressing them in BL21(DE3). Bovine serum albumin (BSA), human serum albumin, human glutathione-S-transferase, and protein A from *Staphylococcus aureus* were purchased from Sigma.

For DELFIA® experiments, plasmids encoding CYP2D6 and AGPR-1 were purchased from Invitrogen (Ultimate™ Human ORF Clones), subcloned in *E. coli* strain DH5 $\alpha$ , and expressed in BL21(DE3). All the corresponding proteins were purified via affinity chromatography on IMAC resin.

**Protein Quality Control**—Purified recombinant proteins obtained as described above were analyzed via SDS-PAGE (Criterion PAGE system Bio-Rad) followed by Coomassie Blue staining of the gels to assess their integrity and purity (Supplemental Fig. S1). Protein purity was assessed via BioRad ChemiDoc™ XRS, with Quantity One® software. Proteins showing purity levels > 70% were used for protein array preparation.

To further analyze the quality of the purified proteins, we performed Western blot analysis on the purified proteins with an anti-His monoclonal antibody (mAb). The proteins were resolved on 4% to 12% precast SDS-PAGE gradient Tricine gels under reducing conditions and electroblotted onto nitrocellulose membranes (Bio-Rad) according to the manufacturer's instructions. The membranes were blocked with 5% nonfat milk in PBS with 0.1% Tween 20 (TPBS) for 1 h at room temperature, incubated with  $\alpha$ -His mAb (GE Healthcare) diluted 1:1000 in 3% nonfat milk in TPBS for 1 h at room temperature, and washed three times in TPBS. The secondary HRP-conjugated antibody ( $\alpha$ -mouse immunoglobulin/HRP) (GE-Healthcare) was diluted 1:1000 in 3% nonfat milk in TPBS and incubated for 1 h at room temperature. The proteins were visualized by means of enhanced chemiluminescence (Super Signal West Pico Chemiluminescence Substrate) (Thermo Scientific) and detected with LAS-3000 (Fujifilm, Wayne, NJ).

**Protein Microarray Printing**—Protein microarrays were generated by spotting the 1626 affinity-purified recombinant proteins (0.5 mg/ml in 6 M urea) in four replicates on nitrocellulose-coated slides (FAST slides) (GE Healthcare) using Stealth SMP3 spotting pins (TeleChem International, Sunnyvale, CA) and a Microgrid II microarray contact printer (Biorobotics, Veldzigt, The Netherlands), resulting in spots with a diameter of ~130  $\mu$ m. As an experimental positive control, a curve of human IgG at 11 different concentrations (from 0.001 to 1 mg/ml) was spotted on the arrays in eight replicates (in 6 M urea) (Supplemental Fig. S3A). Several spots of buffer alone were also printed and used to assess possible nonspecific signals due to cross contamination. A quality control of the spotting procedure was performed on 10% of randomly chosen slides. The percentage of proteins successfully spotted on the slides was assessed by hybridizing the arrays with an  $\alpha$ -His mAb followed by an Alexa-647 conjugated  $\alpha$ -human IgG secondary antibody and estimating the number of spots with a mean fluorescence intensity (MFI) value significantly above background. A distance matrix was calculated using TIGR Multiexperiment Viewer (version MeV4.7) software (13) to evaluate the system reproducibility (Supplemental Fig. S3B). The spotted microarrays were allowed to remain at room temperature for 1 h before being stored at 4 °C until use.

**Incubation and Scanning of Protein Microarray**—Incubation was automatically performed with a TECAN hybridization station (HS

4800™ Pro) (TECAN, Salzburg, Austria). The microarray slides were prewashed for 3 min in TPBS and saturated with BlockIt™ microarray blocking buffer (Arrayit Corporation, Sunnyvale, CA) for 45 min at 25 °C under mild agitation. After the injection of 105 µl of diluted human serum (1:300 in blocking buffer with 0.1% Tween 20), microarrays were incubated at 25 °C for 45 min with gentle agitation. The microarrays were then washed in TPBS at 25 °C for three cycles of 1 min (wash time) and 30 s (soak time).

After that, microarray slides were incubated for 1 h at 25 °C with Alexa-647-conjugated  $\alpha$ -human IgG (Invitrogen) (1:800 in blocking buffer) in the dark. The microarrays were then washed at 25 °C two times in TPBS (1 min wash time, 30 s soak time), two times in PBS (1 min wash time, 30 s soak time), and finally one time in milliQ sterile water (15 s).

The slides were finally dried at 30 °C under nitrogen for 2 min and scanned using a ScanArray Gx PLUS (PerkinElmer, Shelton, CT). 16-bit images were generated with ScanArray™ software at a resolution of 10 µm per pixel and analyzed using ImaGene 8.0 software (Biodiscovery Inc., Hawthorne, CA). A 635 nm laser was used to excite the Alexa-647 dye. The fluorescence intensity of each spot was measured, signal-to-local-background ratios were calculated using ImaGene, and spot morphology and deviation from the expected spot position were considered using the default ImaGene settings.

**Microarray Data Analysis**—For each sample, the background subtracted MFI of replicated spots was determined and subsequently normalized on the basis of the human IgG curve to allow the comparison of data from different experiments (14). Briefly, the MFI values of IgG, spotted at different concentrations (Supplemental Fig. S3A), were fitted by a sigmoid curve, using a maximum likelihood estimator (38). The experimental average IgG curve of each slide was adjusted on the reference sigmoid IgG curve, and the background-subtracted MFI values of each protein were normalized accordingly. On the basis of these results, a normalized MFI value of 4.000 (value corresponds to the normalized MFI value of negative controls (BSA, HSA, Hu-GST) plus 2 standard deviations) was chosen as the lowest signal threshold for scoring a protein as positively recognized by human sera. For each protein, a coefficient of variation (CV%) was calculated on four replicate spots for intra-assay reproducibility (14). Each antigen was checked for displaying a CV% correlated to its MFI on the basis of standard IgG curves. If the CV% value was not within the expected range, the antigen was not considered for further analysis.

Recognition frequency was defined as the percentage of sera reacting with a particular antigen in protein array with an MFI  $\geq$  4.000, and it was calculated for each group of sera. TIGR Multiexperiment Viewer (version MeV4.7) software (13) was used to perform an unsupervised bidimensional hierarchical clustering.

**In-gel Enzymatic Digestion and MALDI-TOF Mass Spectrometry Analysis**—The identity of selected candidate autoantigens was further confirmed by means of tandem mass spectrometry (MS/MS). Protein spots were excised from the gels, destained with 50 mM ammonium bicarbonate (Fluka Chemie AG, Buchs, Switzerland) in 50% acetonitrile (Mallinckrodt Baker), dehydrated once with pure acetonitrile, and air-dried. Dried spots were digested for 2 h at 37 °C in 12 µl of 0.012 µg/µl sequencing grade modified trypsin (Promega, Madison, WI) in 5 mM ammonium bicarbonate. After digestion, 0.6 µl were loaded on a matrix PAC target (Prespotted AnchorChip 96, set for proteomics) (Bruker Daltonics, Bremen, Germany) and air-dried. Spots were washed with 0.6 µl of a solution of 70% ethanol and 0.1% trifluoroacetic acid. Analysis was performed using an Ultraflex III matrix assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometer (Bruker Daltonics). Peptides were selected in the mass range of 900–3500 Da. Also, MS/MS spectra were acquired and externally calibrated by using a combination of standards pre-spotted on the target (Bruker Daltonics). MS and MS/MS spectra were ana-

lyzed with FlexAnalysis (version 3.0) (Bruker Daltonics). Search parameters were as follows: variable modifications, carbamyl (N-term), oxidation (Met); cleavage by trypsin (cuts C-terminal side of Lys and Arg unless the next residue is Pro); mass tolerance, 300 ppm; missed cleavage, 0; mass values, MH<sup>+</sup> monoisotopic. Monoisotopic peaks were annotated with FlexAnalysis default parameters and manually revised. The peptide sequence was determined with Mascot software run on a public database (NCBI nr *Homo sapiens*, release 20100616, or SwissProt *Homo sapiens*, release 2010\_07, # 536789 sequence entries). The accession number, annotation, Mowse score, percentage of protein coverage, number of unique peptides matched, and number of masses not matched are reported in the Supplemental data.

**Dissociation-enhanced Lanthanide Fluorescence ImmunoAssays**—The Dissociation-enhanced Lanthanide Fluorescence ImmunoAssay (DELFI)® is a time-resolved fluorescence method that can be used to study antibody binding to solid-phase proteins or peptides. The purified recombinant proteins were used at a concentration of 20 µg/ml (15) in 6 M urea to coat DELFI® plates (PerkinElmer). Plates were then blocked for 1 h at 37 °C with a blocking reagent (PerkinElmer). The serum samples, diluted 1:300 in PBS with 1% BSA (Sigma) and 0.1% Tween 20 (Sigma), were incubated on the plates for 1 h at 37 °C. Plates were then washed five times with washing buffer (PerkinElmer) and incubated for 30 min at room temperature in the dark with europium-labeled  $\alpha$ -human IgG serum (1:500 in diluting buffer) (PerkinElmer). After extensive washing, plates were left at room temperature for 10 min and then read on an Infinite F200 PRO instrument (Tecan).

Fluorescence intensity values higher than the mean of buffer plus 3 standard deviations were considered as positive.

**Surface Staining of IL4R on HeLa Cells**—To assess the recognition of native IL4 receptor (IL4R) by human sera, full length IL4R was overexpressed in HeLa cells. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. The human cDNA clone of full length IL4R, transcript variant 1, was purchased from OriGene Technologies (Rockville, MD). HeLa cells were transfected with Lipofectamine 2000 Transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded at  $5 \times 10^5$  cells/well in 24-well dishes and left overnight in medium without penicillin-streptomycin. 1 µg of plasmid DNA and 2 µl of Lipofectamine 2000 reagent were diluted in 100 µl of Opti-MEM (Invitrogen) and pre-incubated for 20 min to allow DNA-Lipofectamine complexes to form; cells were then incubated with Opti-MEM (Invitrogen) containing DNA-Lipofectamine complexes at 37 °C for 16 h. After that, Opti-MEM was replaced with fresh DMEM and, at 24 h post-transfection, cells were assayed for IL4R expression. For surface staining, cells were non-enzymatically detached, harvested, and washed in PBS. To assess IL4R expression on the cell surface,  $1 \times 10^5$  cells were incubated with PE-conjugated anti-CD124 (Beckman-Coulter, Krefeld, Germany) and with matched mouse Isotype as a control (1:10 in PBS 1% BSA) for 30 min at 37 °C. Non-transfected cells were used as a control. To assess sera reactivity to IL4R expressed on HeLa cells,  $1 \times 10^5$  transfected cells were incubated with human sera (1:2 in PBS 1% BSA) followed by PE-conjugated anti-Human IgG (1:200 in PBS 1% BSA) for 30 min at 37 °C. Cells stained with secondary antibody alone were used as a control. IL4R staining was analyzed by means of flow cytometry with a FACS Canto II analyzer (BD Biosciences), and data were processed with the program FlowJo (flow cytometry analysis software).

**STAT6 Phosphorylation Assay**—To assess the inhibition of IL4-mediated Stat6 phosphorylation by patient sera, peripheral blood mononuclear cells were isolated from healthy donor blood via density gradient centrifugation (Ficoll). CD4-positive T cells were magnetically separated (CD4+T Cell Isolation Kit II, Miltenyi Biotec, Bergisch Gladbach, Germany), resuspended in RPMI medium (Invitrogen) with



10% fetal calf serum, and seeded in 96 well plates at  $1 \times 10^6$  cells/ml. Cells were pre-incubated for 1 h at 37 °C with sera of patients or healthy donors (1:2 in RPMI) or with neutralizing anti-IL4R goat polyclonal antibody (100 µg/ml) (R&D, Minneapolis, MN) as a control; cells were then stimulated with IL4 (0.2 ng/ml) (Miltenyi Biotec) for 1 h at 37 °C, fixed for 10 min at 4 °C (BD fixation buffer), washed twice in PBS 1% BSA, and permeabilized (BD Perm Buffer III) for 30 min at 4 °C. Intracellular staining with PE mouse anti-Stat6(Y641)P antibody (BD Biosciences) was performed by incubating cells for 30 min at room temperature in the dark. The percentage of Stat6P positive cells was measured by means of flow cytometry with a FACS Canto II analyzer (Becton Dickinson), and data were processed with the program FlowJo (flow cytometry analysis software). To confirm that the mechanism was mediated by antibodies and not by other factors present in the serum, sera from patients and from healthy donors were depleted from anti-IL4R(FNIII) antibodies and tested via Stat6 phosphorylation assay. To do this, 2 mg of purified protein were blotted on nitrocellulose membrane and then incubated with patients or healthy donor sera. After this incubation, sera were collected and tested as described above.

**Soluble IL4R Detection**—The amount of soluble IL4R was determined using an Abcam sIL4R Elisa kit. Briefly, sera were diluted 1:4 in diluent buffer provided in the kit and incubated in 96 well plates coated with anti-sIL4R. Biotinylated monoclonal antibody specific for sIL4R was added to the wells, and plates were incubated for 1 h at room temperature. After washing, streptavidin-HRP was added to the wells, and plates were incubated for an additional 30 min. 3,3',5,5'-tetramethylbenzidine substrate was then added, and the optical density at 450 nm was read on an Infinite F200 PRO instrument (Tecan).

**Statistical Analysis**—Results of protein microarray and DELFIA® experiments were analyzed using the two-tailed  $\chi^2$  test, the Student's *t* test, the Fisher's exact tests, or the analysis of variance test. The Benjamini-Hochberg correction for multiple testing was used for the analysis of microarray data. Statistical analysis was carried out with the use of TIGR Multiexperiment Viewer or GraphPad software. Predictive analysis of microarray (PAM) was performed using the statistical package PAM 1.51 with the statistical tool R (<http://www.stat.stanford.edu/~tibs/PAM/index.html>) (16). PAM executes a sample classification training routine from expression data via the nearest shrunken centroid procedure to find markers that discriminate best between AIH patients and HDs. Data were log-transformed, mean centered, and standard deviation scaled. After training of the PAM classifier, we performed a 10-fold cross validation in order to check the accuracy of the model and better select the threshold as the one giving the lowest misclassification error. To evaluate the performance of autoantigen combinations in discriminating AIH patients from HDs, logistic regression analysis was performed with R. We created logistic regression models with signals of four autoantigens (IL4R(FNIII), AL137145, C17orf99, APCDD1L) or with signals of two known AIH autoantigens as controls (CYP2D6 and AGPR-1). The probabilities were calculated as follows:  $p = \exp((\sum(b_i x_i) + c) / (1 + \sum(b_i x_i) + c))$ , where *p* is the probability of each case, *i* = 1 to *n*, *b* is the regression coefficient of a given autoantigen, *x* is the signal intensity, and *c* is a constant generated by the model. The ROC package was used to obtain the Receiver Operating Characteristic (ROC) curves of the models and the area under the curve (AUC) values (17).

## RESULTS

**Design and Construction of a Microarray with Secreted or Membrane Associated Human Proteins**—To identify self-antigens recognized by antibodies from patients with AIH, we developed a microarray by printing 1626 recombinant products (Supplemental Tables S1 and S2) that corresponded to

TABLE I  
Predicted subcellular localization of the human recombinant proteins used for the microarray construction

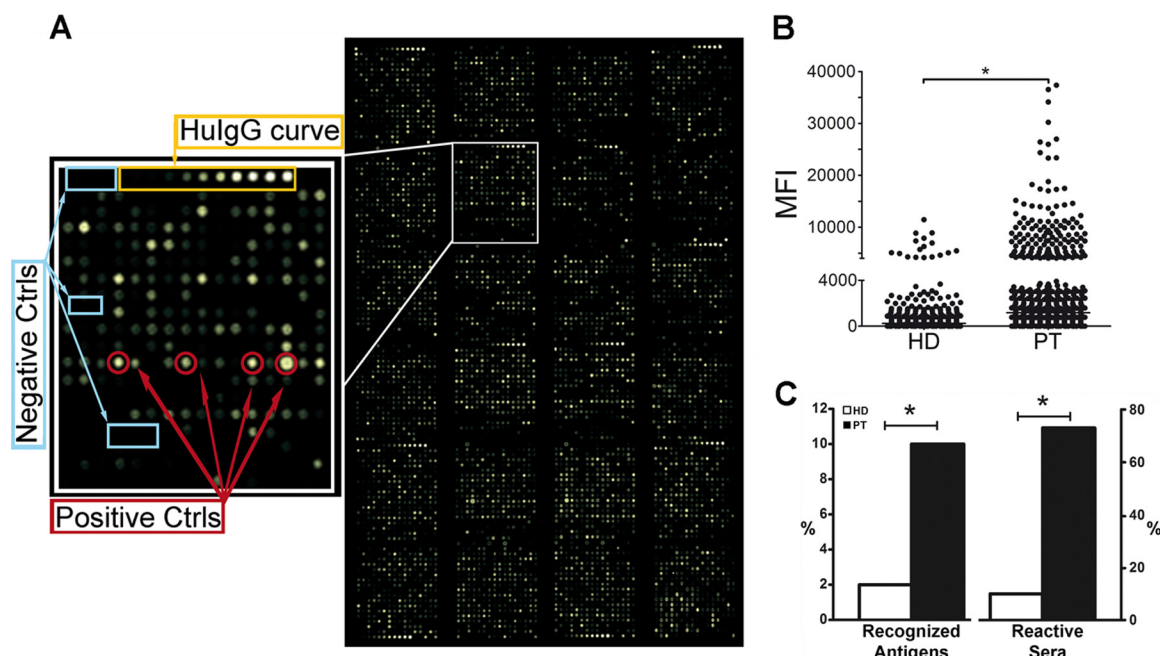
Compartment	Percentage of proteins
Cell membrane	46
Secreted	23
Intracellular membrane	11
Cytoplasm	11
Mitochondrial membrane	4
Nucleus	4
Mitochondrion	1

1371 distinct human proteins (Table I). 1329 of the 1371 proteins were selected through bioinformatic analysis of the whole human genome as hypothetical and/or poorly characterized by the available annotation or published information and carrying either a signal peptide (23% of them) or at least one transmembrane domain (61% of them, with 75% of those assigned to the plasmatic cell membrane and the remaining 25% assigned to mitochondrial or endoplasmic reticulum membranes) (12). Forty-two of the 1371 proteins had a well-known immunological function, with a CD number assigned, and were all surface exposed (Supplemental Table S2).

Proteins were cloned as either full-length products (31%) or protein domains (69%) with an N-terminal histidine tag, expressed in *E. coli*, purified by affinity chromatography and checked for purity and integrity (Supplemental Fig. S1). Protein arrays were prepared by printing four replicates of each protein onto nitrocellulose-covered glass slides, along with several controls (Supplemental Fig. S2). Replicates were randomly distributed to get optimal signal reproducibility. The final protein microarray layout consisted of 24 grids of 304 spots each, for a total of 7296 spots. The quality of the microarrays was assessed by probing 10% of the slides with an anti-His mAb and by determining the number of immobilized proteins with signal intensity significantly above background. About 90% of proteins fulfilled this criterion (data not shown). Moreover, a high correlation among signal intensities of different slides was observed, indicating high experimental reproducibility (Supplemental Fig. S3B). A representative picture of the array is shown in Fig. 1A.

In summary, we obtained high quality microarrays comprising more than 1600 human proteins that can be used as tool for the identification of autoantigens recognized by the sera of patients with any disease of interest.

**Protein Microarray Allows Identification of New Autoantigens Recognized by IgG of Patients Affected by AIH**—In order to identify a panel of autoantigens differentially recognized by patients with AIH (as opposed to HDs), the protein microarrays were probed with a sample set (defined as the discovery set) comprising 15 sera from patients with AIH and 78 sera from healthy donors. The patients' clinical data are summarized in Table II.



**FIG. 1. The microarray made of secreted and membrane proteins highlights a higher immunoreactivity in sera of AIH patients than in that of healthy donors (HDs).** *A*, Representative image of the protein array containing 1626 His-tagged human recombinant proteins (predicted to be secreted or membrane associated) probed with an anti-His mAb. The microarray included 24 grids (like the one shown in the enlarged box), with each one containing 304 protein spots including positive (such as viral and bacterial proteins) and negative (such as buffer or BSA) controls, as well as an IgG calibration curve (HulG curve) for data normalization. *B*, Comparison of mean fluorescence intensity (MFI) of all spotted proteins probed with sera of 78 HDs and 15 AIH patients. Each dot represents the MFI of a single protein. A cut-off value  $\geq 4,000$  was used to score a protein as positive. Asterisk denotes statistical significance (Student's *t* test,  $p < 0.01$ ). *C*, Bars on the left side represent the percentage of autoantigens recognized by more than 15% of AIH and HD sera (threshold is determined on the basis of the average HD recognition); bars on the right side represent the percentage of AIH and HD sera reacting with more than 3% of the array proteins (threshold is determined on the basis of the average HD reactivity). Asterisk denotes statistical significance ( $\chi^2$  test,  $p < 0.01$ ).

TABLE II  
Clinical characteristics of the serum samples used in this study

Group	Abbreviation	Subgroup (n)	n	Source <sup>a</sup>	Age: Mean $\pm$ S.D. (median)	Sex (n)
Discovery						
Healthy donor	HD	—	78	1	44 $\pm$ 10 (45)	F (20) M (58)
Autoimmune hepatitis	AIH	Type 1 (15) Type 2 (0)	15	2	50 $\pm$ 21 (54)	F (13) M (2)
Viral hepatitis	VH	HCV	110	2, 3	55 $\pm$ 15 (56)	F (41) M (69)
Validation						
Healthy donor	HD	—	50	1	43 $\pm$ 8 (43)	F (41) M (9)
Autoimmune hepatitis	AIH	Type 1 (43) Type 2 (4) n.c. (3)	50	2, 4	45 $\pm$ 21 (49)	F (41) M (9)
Viral hepatitis	VH	HCV (50) HBV (24)	74	2, 5	51 $\pm$ 13 (52)	F (28) M (46)

<sup>a</sup> Origin of samples: 1) IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; 2) Sant'Orsola University Hospital, Bologna, Italy; 3) Hepatology Unit, University Hospital, Pisa, Italy; 4) Center for Autoimmune Liver Diseases, IRCCS Istituto Clinico Humanitas, Rozzano, Italy; 5) Center for Systemic Manifestations of Hepatitis Viruses (MaSVE), Firenze, Italy.  
n.c., not classified.

First, we compared the autoreactivity of patient sera to that of HD sera. AIH sera displayed a higher reactivity toward self proteins than HD sera, as documented both by the intensity

(MFI) of recognition signals (Fig. 1B) and by recognition frequencies (Fig. 1C). Autoantigens recognized by AIH sera were then ranked according to (i) the recognition frequency and (ii)

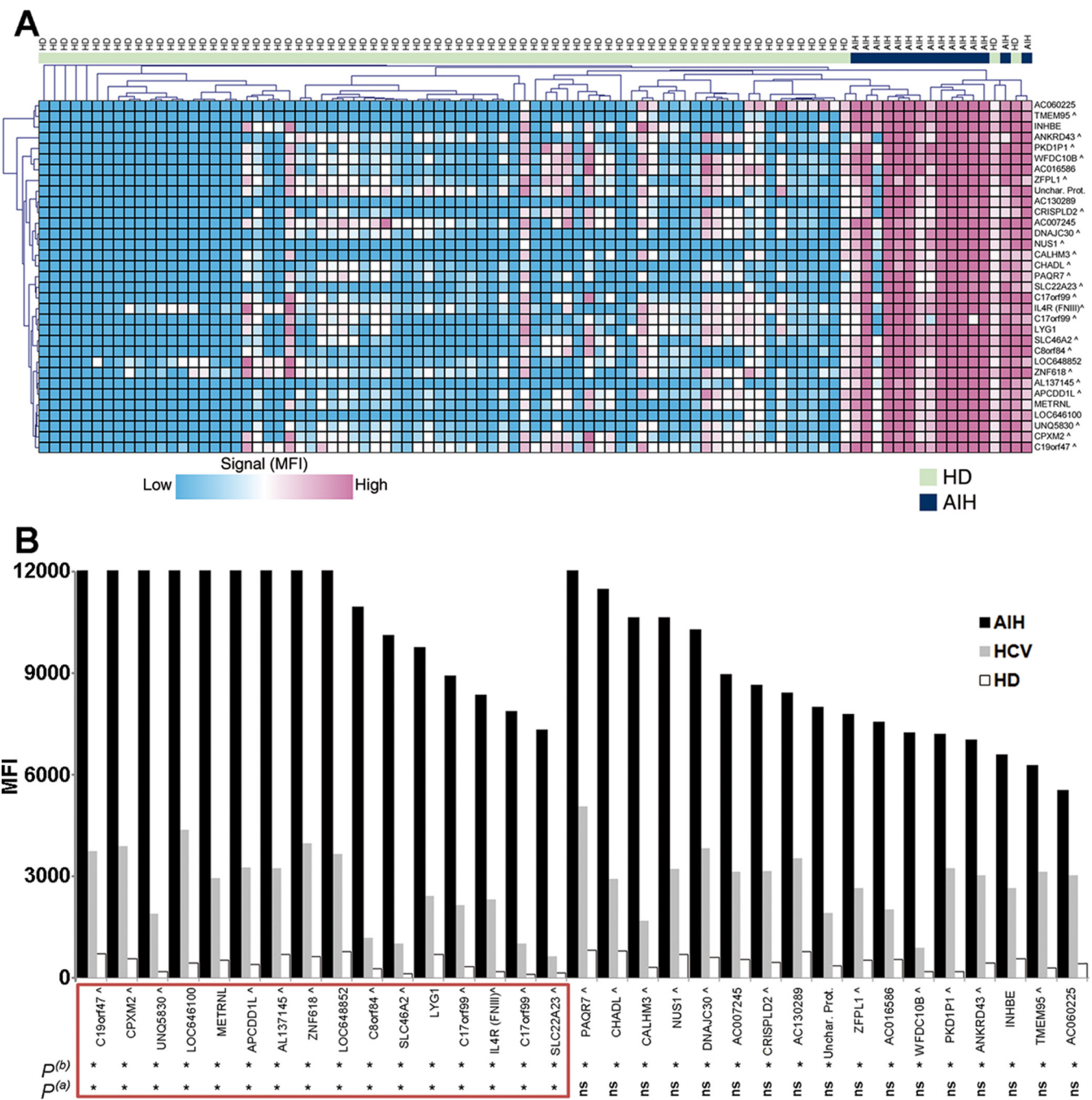
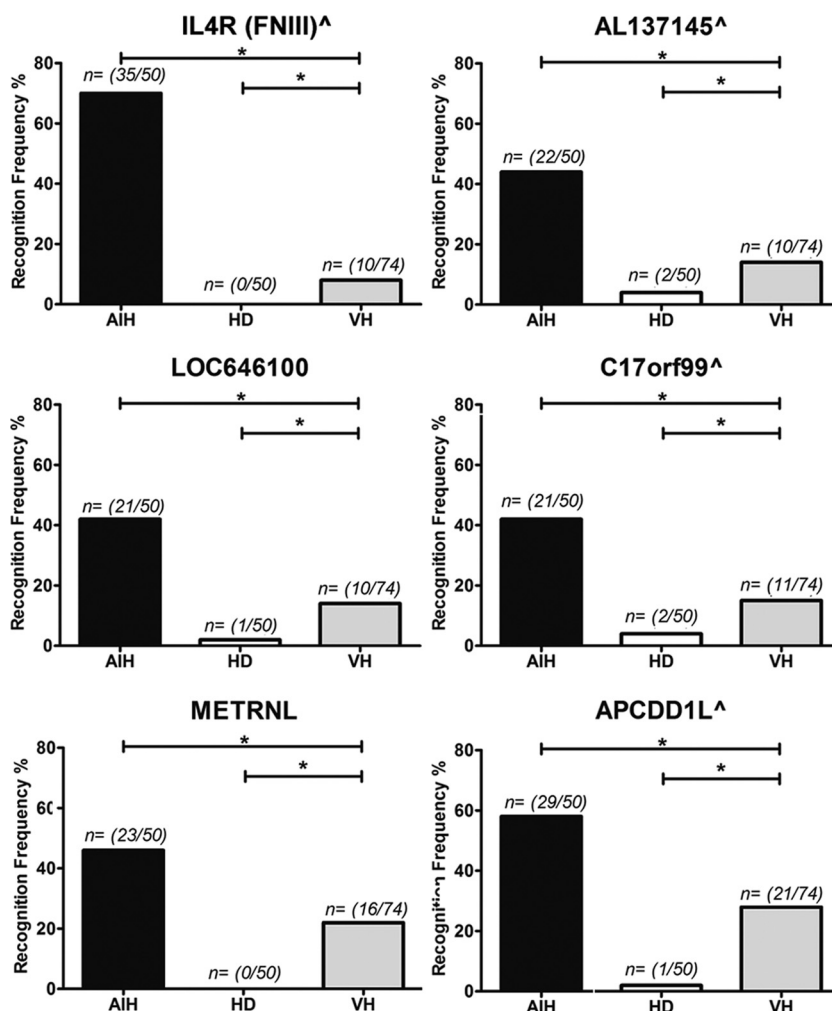


FIG. 2. **Candidate autoantigens specifically recognized by AIH patients as opposed to HDs and HCV patients.** Statistical analyses allowed the selection of 33 proteins that underwent unsupervised hierarchical clustering analysis. **A**, Good discrimination of AIH and HD sera. Sera are arranged in columns, and proteins in rows. **Red** indicates positive immunoreactivity, and **blue** indicates low or no immunoreactivity. **B**, MFI of the autoantigens probed with sera from HD, AIH, and chronic HCV patients. Significant differences were observed for 33 autoantigens between AIH and HD and for 16 autoantigens between AIH and HCV (red box). Asterisks denote  $p < 0.01$  (Student's  $t$  test).  $p^{(a)}$ :  $p$  of AIH versus HCV patients;  $p^{(b)}$ :  $p$  of AIH patients versus HD.  $\Delta$ : Protein cloned and expressed as domain.

the MFI. Self proteins were regarded as potential autoantigens if they were recognized with MFI significantly higher in patients than in HD sera ( $t$  test,  $p < 0.01$ ) and if they were recognized by less than 5% of the HD sera and by more than 50% of patient sera (Fisher test,  $p < 0.01$ ). In this way we identified 33 proteins that allowed good discrimination of the

two populations of sera, as shown in the unsupervised hierarchical clustering analysis in Fig. 2A. We then asked whether sera from patients with HCV liver disease displayed the same autoreactivity pattern. We therefore tested the same microarray with sera from 110 patients with chronic HCV infection (Table II). Fig. 2B shows the MFIs of the 33 autoantigens with





**FIG. 3. Six proteins are confirmed as AIH autoantigens in DELFIA® assay.** Recognition frequency of the best six autoantigens as determined by DELFIA®. Proteins were tested with sera from AIH patients ( $n = 50$ ), healthy donors ( $n = 50$ ), and HBV or HCV viral hepatitis (VH) patients ( $n = 74$ ). Each serum was tested three times in independent experiments. \*: Statistical significance (Fisher exact test,  $p < 0.01$ ). <sup>Δ</sup>: Protein cloned and expressed as domain.

sera from AIH, HD, and HCV and indicates that 16/33 autoantigens reacted preferentially and significantly with sera from AIH patients ( $t$  test,  $p < 0.01$ ).

In order to confirm the identities of the proteins, we resolved the 16 recombinant autoantigens of interest (Supplemental Table S3) via SDS-PAGE, and the prevalent bands were excised from gels, digested with trypsin, and subsequently analyzed via MALDI-TOF mass spectrometry. All antigen identities were confirmed by either peptide mass fingerprint or fragmentation of selected ions (details, Mascot scores, and MS spectra are provided as Supplemental Data).

We therefore concluded that 16 autoantigens, identified via protein microarray, are differentially recognized by AIH patients as opposed to HDs and chronic HCV patients.

**Validation of Selected Autoantigens with an Independent Sample Set of Sera Confirms 6 of the 16 Proteins are New Potential AIH Biomarkers**—In order to validate protein microarray results with a different assay and larger panels of patients sera, we used DELFIA® to screen an independent validation set of sera comprising 50 AIH patients, 50 HDs, and 74 patients with chronic viral hepatitis (50 HCV and 24 HBV)

(Table II). DELFIA® assay was therefore used to assess the IgG response both to the 16 autoantigens that were selected with the discovery set and to CYP2D6 and AGPR-1, two benchmark protein autoantigens in AIH (18, 19).

All 16 antigens displayed higher MFI than HD and chronic viral hepatitis patients (Supplemental Fig. S4), and 6 of these 16 antigens displayed significantly higher recognition frequency by AIH patients than by HDs and viral hepatitis patients (Fig. 3). These six antigens showed high sensitivity (from 42% to 70% of positive AIH patients) and specificity (from 96% to 100% of negative HDs). Interestingly, individual sensitivity was comparable to that obtained in our assay with CYP2D6 and AGPR-1, two benchmark protein autoantigens in AIH, whereas individual specificity was higher for our six candidates (Table III).

We then performed a classification of the validation samples with a nearest shrunken centroid algorithm (PAM), which identified a minimal set of five predictors (Supplemental Fig. S5), and classified AIH samples with an accuracy of 94%. Importantly, these classifiers corresponded to the top antigens previously sorted out on the basis of the sensitivity and

TABLE III  
Sensitivity (S.E.) and specificity (SP) of individual antigens identified as candidate AIH biomarkers

Description	Protein ID	Combo <sup>a</sup>	SE (%)	SP (HD) (%)	SP (VH) (%)
Asialoglycoprotein receptor	AGPR-1		56	82	47
Cytochrome P4502D6	CYP2D6		62	72	45
Interleukin-4 receptor domain	IL4R (FNIII) <sup>b</sup>	●	70	100	86
Putative uncharacterized protein	AL137145 <sup>b</sup>	●	44	96	86
Putative uncharacterized protein	LOC646100		42	98	86
Uncharacterized protein C17orf99	C17orf99 <sup>b</sup>	●	42	96	85
Meteorin-like protein precursor	METRNL		46	100	78
Protein APCDD1-like precursor	APCDD1L <sup>b</sup>	●	58	98	72

<sup>a</sup> Antigens used for combination assay.  
<sup>b</sup> Protein expressed as domain.  
HD, healthy donors; VH, chronic B or C viral hepatitis patients; SE % = percentage of positive AIH sera; SP % = percentage of negative HD or VH sera; IL4R (FNIII), interleukin-4 receptor fibronectin type III domain.

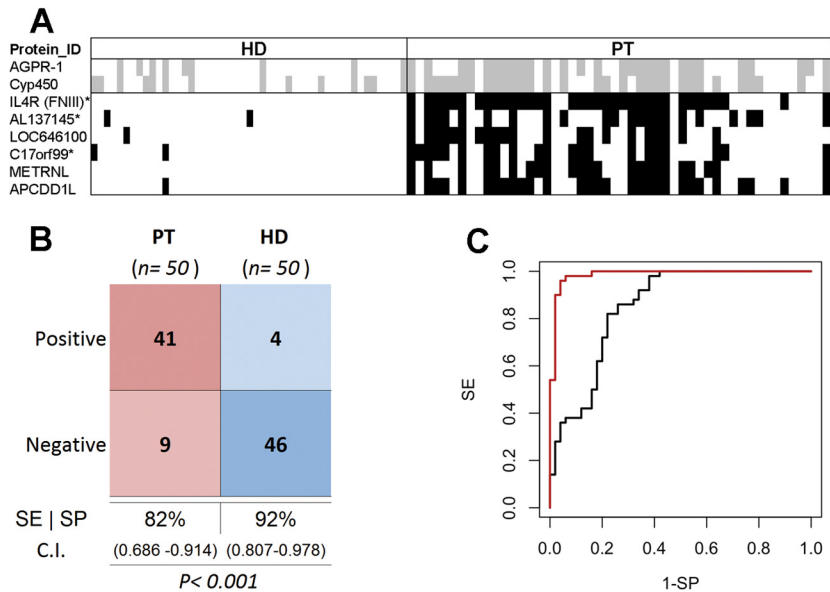


FIG. 4. **Combination of four of the six autoantigens identifies AIH patients with high sensitivity and specificity.** A, Recognition of the six identified autoantigens by validation sera samples. Asterisk indicates protein cloned and expressed as domain. B, Ability to discriminate AIH from HD by combination of four antigens, IL4R(FNIII), AL137145, C17orf99, and APCDD1L. Numbers in boxes indicate AIH and HD sera that recognize (positive) or do not recognize (negative) at least one of the four antigens. Sensitivity (SE) and specificity (SP) with 95% confidence intervals (C.I.) are indicated below the panel. *p* values are calculated with  $\chi^2$  tests. C, Logistic regression models for the combination of the four autoantigens (red curve) and the two known control proteins (AGPR-1, CYP2D6) (black curve) were calculated and represented as ROC curves. The AUC values are 0.99 for the four new antigens and 0.85 for the known controls.

specificity values, validating the selection of these proteins as best antigens for further characterization.

We next assessed the discrimination power of combinations of the autoantigens. Fig. 4A shows the seroreactivity of the validation sample set to all six autoantigens and reveals that two of the antigens (LOC646100 and METRNL) have reactivity that overlaps with the IL4R(FNIII) domain. We therefore assessed whether the combination of only four antigens (IL4R(FNIII), AL137145, C17orf99, APCDD1L) performed better than individual antigens. Fig. 4B shows that the four-antigen combination achieved 82% sensitivity and 92% specificity, thus performing better than the individual antigens and the combination of the two known autoantigens AGPR-1 and CYP2D6 (SE = 68% and SP = 68%). Moreover, Fig. 4C

compares the ROC curves of logistic regression models obtained with the combination of our four autoantigens and a combination of the two known autoantigens, and it indicates that our four-autoantigen combination is superior to the combination of AGPR-1 and CYP2D6.

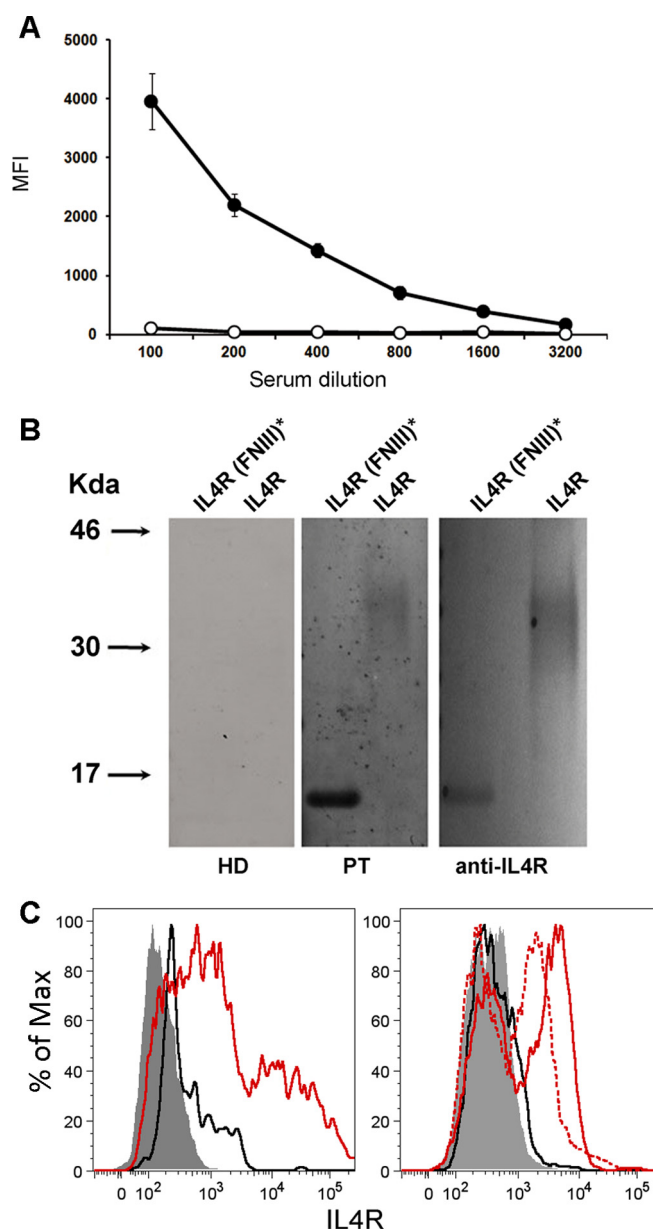
**IL4R is the Target of Autoantibodies Capable of Neutralizing IL4 Activity**—To address the biological significance of the newly identified autoantigens, we checked the public databases for any information regarding their putative function. We found that five of the six autoantigens have poorly known functions. Indeed, three of them (AL137145, LOC646100, and C17orf99) are completely uncharacterized secreted proteins; METRNL is a secreted protein annotated as a meteorin-like protein precursor, and APCDD1L is a



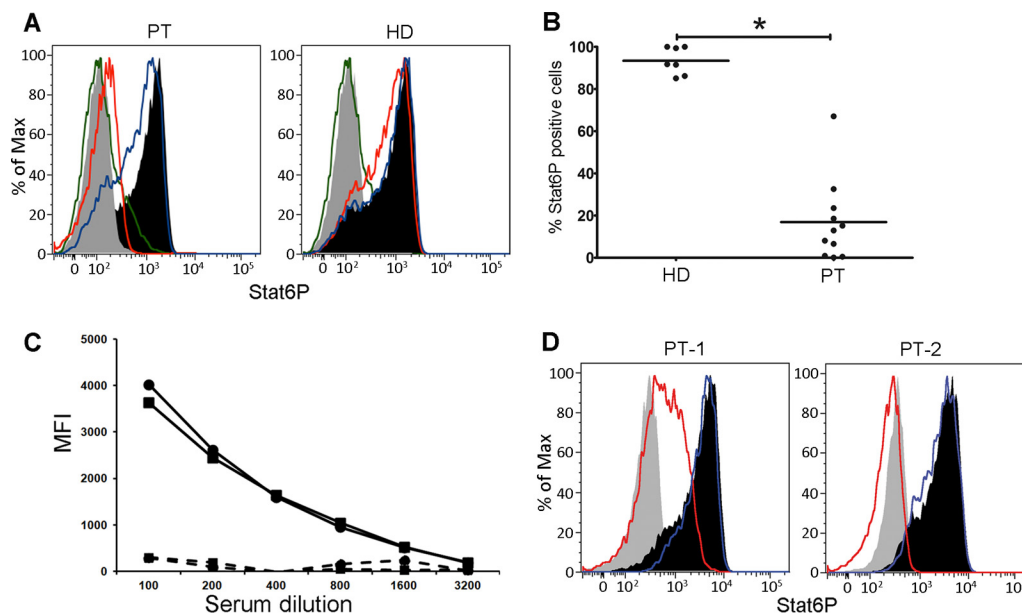
membrane protein annotated as a protein APCDD1-like precursor (Adenomatosis polyposis coli down-regulated 1 protein-like). One of the proteins is instead a domain of a well-known membrane protein, as it corresponds to the FNIII domain of the IL4R  $\alpha$  chain (IL4R or CD124), which is expressed in lymphocytes in association with cytokine receptor common gamma chain (Type I IL4R), as well as in hepatocytes in association with the interleukin 13 receptor  $\alpha$ 1 chain (Type II IL4R) (20). Interestingly, as shown in Table III, IL4R(FNIII) is the antigen displaying the highest individual sensitivity (70%) and specificity (100%) for AIH. Therefore, we decided to focus our attention on the possible functional role of autoantibodies targeting IL4R.

Firstly, we confirmed antibody specificity by titrating sera from four different patients who displayed high reactivity for CD124 and found they were able to recognize the receptor domain in up to a 1:800 dilution (Fig. 5A). Secondly, we asked how patient sera compared with commercially available neutralizing anti-CD124 goat polyclonal antibody in terms of the ability to recognize both the human CD124 domain that we used in our protein array and a recombinant form of the human CD124 expressed in insect cells. Fig. 5B shows that patient sera and the goat anti-CD124 antibodies both recognized the two forms of CD124 in Western blot analysis. Finally, we assessed the capability of human sera to recognize the native IL4R when expressed on a mammalian cell surface. We therefore overexpressed full-length IL4R in HeLa cells and then incubated cells with sera of AIH patients and HDs and with anti-CD124 antibody as a control. Fig. 5C shows that the sera of patients, but not of HDs, recognized native IL4R on the HeLa cell surface.

Given that the FNIII domain of CD124 is involved in the interaction with IL4, we then asked whether these autoantibodies neutralized the interaction of IL4 with its receptor. As the binding of IL4 to its receptor results in the specific phosphorylation of Stat6, we assessed by means of flow cytometry whether patient sera inhibited IL4-mediated Stat6 phosphorylation. Figs. 6A and 6B show that when cells expressing CD124 are pre-incubated with sera from AIH patients, but not with control sera, there is a dramatic reduction of the Stat6 phosphorylation that follows exposure to IL4. To confirm that the inhibition of Stat6 phosphorylation was an antibody-mediated mechanism, sera depleted of anti-IL4R (FNIII) antibodies were tested for their ability to block IL4 signaling. Fig. 6A shows that sera depleted of anti-IL4R antibodies no longer inhibited IL4-induced Stat6 phosphorylation. Thus we have demonstrated that AIH patient sera neutralize IL4 *in vitro* because of anti-IL4R antibodies. Noteworthy, the inhibition of Stat6 phosphorylation correlated with the signal intensity of the anti-IL4R reactivity we detected in the patient sera via DELFIA® (Supplemental Fig. S6), and it is dilution dependent (Supplemental Fig. S7). To rule out that the neutralization of IL4 activity observed with patient sera was due to a competitive effect of a circulating soluble form of IL4R (sIL4R) (21), we



**FIG. 5. AIH sera recognize IL4 receptor (CD124).** A, Sera from four AIH patients (black circles) and four HDs (white circles) were titrated for recognition (MFI) of IL4R (FNIII) in DELFIA® assays. Error bars: standard error. B, A representative Western blot experiment (out of three) shows either sera from AIH patients and HDs or a neutralizing anti-CD124 polyclonal antibody ( $\alpha$  IL4R) reacting with IL4R domain or a recombinant form of CD124 (IL4R) expressed in insect cells. Recombinant IL4R domain, expressed in *E. coli*, migrates as a single band of 11 kDa. The IL4R due to heterogeneous glycosylation appears as a smear ranging from 30 to 35 kDa. C, Left panel shows the surface staining of HeLa cells (black line) and HeLa cells overexpressing IL4R (red line) with anti-CD124. Mouse Ig isotype was used as a control (solid gray line). Right panel shows a representative result of surface staining of HeLa cells overexpressing IL4R with sera from AIH patients (results from two patients are shown as solid or dashed red lines) or healthy donor (black line) followed by secondary PE-conjugated anti-HulG antibody. Cells stained with secondary antibody alone were used as a control (solid gray line).



**FIG. 6. Neutralization of IL4 activity by AIH patient sera.** A, A representative experiment shows that patients' sera (left panel) completely inhibit Stat6 phosphorylation induced by recombinant IL4 on human CD4<sup>+</sup> T lymphocytes, whereas HD sera (right panel) do not. The same patient serum, when depleted of anti-IL4R(FNIII) antibodies, lost its capacity to inhibit Stat6 phosphorylation (left panel, blue line). Black solid line: cells incubated with IL4; gray solid line: cells incubated with medium alone; green line: cells incubated with IL4 and a control neutralizing anti-IL4R polyclonal antibody; red line: cells incubated with IL4 and human sera, PT or HD; blue line: cells incubated with IL4 and human sera, AIH or HD, after depletion of anti-IL4R(FNIII) antibodies. B, Scatter plot shows percentage of Stat6 phosphorylation induced by IL4 in the presence of sera from AIH patients (11 individuals) or HDs (7 individuals). Each serum was tested twice in duplicate. Statistics: Student's *t* test ( $p < 0.01$ ). C, DELFIA<sup>®</sup> assay showing titration of IL4R(FNIII) recognition by two patient sera (●, PT-1; ■, PT-2) collected before (solid lines) and after (dashed lines) immunosuppressive therapy. D, The same sera were tested for their ability to inhibit Stat6 phosphorylation. Black solid line: cells incubated with IL4; gray solid line: cells incubated with medium alone; red line: cells incubated with IL4 and PT sera collected before therapy; blue line: cells incubated with IL4 and PT sera collected after therapy.

used ELISA assays to test for the possible presence of sIL4R in the sera of 20 AIH patients and 20 HDs. No significant differences in sIL4R were observed in the sera of patients and HDs (Supplemental Fig. S8). Interestingly, sera from AIH patients under immunosuppressive therapy showed a strong reduction of anti-CD124 antibodies as measured by both quantitative titer assessment with DELFIA<sup>®</sup> (Fig. 6C) and qualitative neutralization of Stat6 phosphorylation (Fig. 6D).

From all of the above, we conclude that patients with AIH have autoantibodies to CD124 and that these antibodies neutralize IL4 signaling.

#### DISCUSSION

This study illustrates the steps and outcome of a custom protein array approach to tackling new autoantigens in AIH. Using an array of about 1600 human recombinant products, we report the identification of several human proteins recognized by autoantibodies that are present in the sera of patients with AIH. We suggest that these autoantibodies might serve both for the improvement of diagnoses and for the development of new immunotherapeutic agents that could interfere with these autoantibodies. In particular, antibodies to IL4R, the autoantigen recognized with the highest sensitivity and specificity by AIH patient sera, inhibit IL4 signal transduction, which demonstrates that these autoantibodies are func-

tional, suggesting a pathogenetic role for the inhibition of IL4 signaling and possibly opening new therapeutic perspectives for AIH.

In recent years, microarrays have become precious tools for biomedical research because they are very suitable for screening great numbers of samples with a very small amount of biological material in a very short time (22). A limitation of protein microarrays is that because of labor intensive protein production processes, they often cover only defined protein families with known relevance to a given scientific question, although important efforts to overcome this limitation have been made recently, and arrays with thousands of proteins are now available (11, 23). In this study we printed our custom array with a relatively small group of proteins that are known to play key roles in the generation and differentiation of immune responses in health and disease, in combination with a functionally unbiased expression library. Our custom protein microarray is made of a library of more than 1600 recombinant products that were expressed in *E. coli* because of the costs and the production and purification issues related to the handling of thousands of proteins. The expression of human proteins in bacteria is not ideal for functional studies, because post-translational modifications are generally lost and because proteins are mostly recov-

ered in denaturing conditions. However, we aimed at identifying polyclonal antibodies specific for linear epitopes of human proteins, as it has been reported that linear epitopes are often recognized by autoantibodies in many autoimmune diseases (24, 25).

The proteins printed in our microarray are recombinant products that are either membrane-associated or secreted proteins, the great majority of which are poorly characterized on the basis of current annotation in public databases and scientific publications (12). The rationale for the use of this specific protein subset was that we were interested in a functionally unbiased search of new autoantigens among thousands of proteins; in addition, we wanted to focus on a functionally well-known small group (40) of cell-bound proteins that are exposed to the extracellular environment and that play crucial functions in regulating immune responses in health and diseases. It is worth noting that although membrane-associated and secreted proteins play a crucial role in many cell recognition and communication processes (26), most of the autoantigens identified so far in autoimmune diseases are intracellular components (11, 27).

We used this protein microarray to screen a panel of sera from patients with AIH, a disease for which there is an unmet medical need for both new and highly specific biomarkers and for more specific therapies. Indeed, the diagnosis of AIH is a complex process carried out via the exclusion of other factors leading to chronic hepatitis (including viral, toxic, genetic, and metabolic causes) and by detecting autoantibodies in indirect immunofluorescence assays on tissue sections from rodents. However, this technique has several limitations, the most significant of which is the strong dependence on operator expertise (6). Moreover, serological overlap of AIH with other liver diseases (such as chronic viral hepatitis or drug-induced hepatitis) (10) is frequently observed. The distinction between autoimmune and viral hepatitis is also important from a therapeutic point of view, because the immunosuppression used in AIH can increase virus replication in chronic viral hepatitis, and treatments used to eradicate viral infection, such as interferon-alpha, might lead to the exacerbation of AIH (10).

Here we show that sera from patients with AIH recognize with high specificity and sensitivity five self proteins of unknown function and one known self protein (IL4R or CD124). This recognition pattern was first detected via protein array on 15 patient sera and subsequently was confirmed in a 96-well plate assay with sera from 50 patients with AIH, whereas sera from HDs and from patients with chronic viral hepatitis did not show significant recognition of these antigens. This autoreactivity pattern was mainly observed in type 1 AIH. Indeed, the great majority of AIH sera tested (100% of sera used in the discovery phase and 86% of sera used in the validation phase) were type 1 AIH, as reported in Table II.

Interestingly, although the individual performance of our new autoantigens for AIH was comparable to that we obtained with CYP2D6 and AGPR-1, two benchmark protein

autoantigens and a combination of four of the six autoantigens were superior in terms of sensitivity and specificity to the benchmark autoantigens alone or in combination. Indeed, we achieved an accuracy of 87% (SE = 82% and SP = 92%) in discriminating AIH patients from HDs with the new antigens, compared with the 68% accuracy of the combined benchmarks (SE = 68%, SP = 68%). Moreover, we achieved an accuracy of 68% (SE = 82% and SP = 61%) in discriminating AIH from viral hepatitis patients, compared with the 46% accuracy of the combined benchmarks (SE = 68%, SP = 31%) (data not shown). Finally, compared with the work of Song and colleagues (11), who recently reported the identification via protein array of three new AIH autoantigens with similar performances, we used an ELISA-like approach to validate our candidates that could be easily translated into standard laboratory practice.

Therefore, we set the stage for the development of a new serological assay that is easy to perform, is highly specific for AIH, and could significantly contribute to improved AIH diagnosis.

Of the five proteins with unknown function, one is a membrane protein (APCDD1L) and four are secreted proteins (AL137145, LOC646100, C17orf99, METRNL). The one known autoantigen is the FNIII domain of the alpha chain of the IL4R (or CD124). Obviously we concentrated our functional investigation on autoantibodies targeting this well-known receptor, but studies are in progress to address the structure and function of the other five poorly known autoantigens.

IL4 is a cytokine mainly produced by CD4<sup>+</sup> Th2 lymphocytes, basophils, mast cells, and eosinophils (20). It plays a key role in several aspects of lymphocyte differentiation and function and has been described as being involved in many autoimmune and inflammatory diseases (28). In particular, IL4 is reported to inhibit Th1 and Th17 differentiation, which are T cell subsets implicated in many autoimmune diseases, including AIH (29, 30). Altered IL4 expression has been reported in several liver diseases, including chronic hepatitis C, drug-induced hepatitis, and disease related to liver transplantation (31). However, its exact pathogenetic role in these diseases is still controversial: for some authors it has a protective effect (32, 33), whereas others have reported that higher IL4 expression in the liver is detrimental, causing hepatocyte apoptosis (34, 35).

IL4 exerts its action by binding its receptor (CD124), which is present on many cell types, including lymphocytes and hepatocytes (20), and activating specific signaling cascades. The IL4R consists of a signaling alpha chain that binds IL4 with high affinity and a trans-activating low affinity chain that can be, according to the cell type, the common gamma chain (immune system cells) or the IL13 receptor alpha1 chain (epithelial cells). After IL4 binding to both immune and epithelial cells, the two chains form a heterodimer and initiate a phosphorylation cascade, which ends up in the specific activation of Stat6. Upon phosphorylation on Tyr641, Stat6 translocates



to the nucleus and in turn activates the transcription of specific genes (36).

Interestingly, the FNIII domain of the CD124 alpha chain, which we found to be recognized with very high frequency (70%) by AIH patient sera, contains two binding sites for IL4 (37). Indeed, our data show that not only does AIH patient sera recognize IL4R, but also IL4-mediated Stat6 phosphorylation is inhibited *in vitro* by AIH patient sera but not by HD or HCV sera. This finding points to the importance of the IL4 pathway in AIH and to the pathogenetic role that autoantibodies against the IL4R might play in AIH. Such antibodies might neutralize IL4 activity directly on hepatocytes and thus interfere with a potential anti-inflammatory role of STAT6, which has been put forward to explain the ability of IL4 to reduce hepatic ischemia/reperfusion injury (32). Autoantibodies that neutralize IL4 activity might favor liver inflammation indirectly by favoring development in lymphoid tissues and recruitment to the liver of T cell subsets, such as Th1 and Th17, involved in autoimmunity and inflammation (30). Indeed, IL4 has been shown in experimental and clinical situations to be capable of ameliorating the effects of tissue-damaging autoimmunity (20). On the other hand, based on data published in the literature (34, 35), one could also theorize that IL4 is detrimental in AIH and that neutralizing anti-CD124 antibodies reflects an attempt of our immune system to buffer a negative role of IL4. Should this be true, immunosuppressive therapy could be detrimental; the sera of patients after steroid treatment display a strong reduction of anti-CD124 antibodies as measured by both DELFIA® and the neutralization of IL4 activity (Fig. 6).

In conclusion, we have described the identification of a new panel of six autoantigens that are very specific and sensitive biomarkers of AIH. In contrast to other, similar approaches (11), our strategy based on a protein array enriched in “external” proteins (*i.e.* proteins physiologically more exposed to the immune system) allowed us to identify autoantigens that not only can be used as diagnostic biomarkers, alone or in combination, but could also give insights into some of the pathogenetic mechanisms involved in this autoimmune disease. Indeed, our results demonstrate that autoantibodies to CD124 (IL4R) have a neutralizing effect on IL4 activity and suggest that these antibodies could have a pathogenetic role by favoring an inflammatory milieu leading to liver damage.

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§ This article contains [Supplemental Figures S1 to S8, Tables S1 and S3, and Data](#).

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